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Superiority of ABTS over *Trinder* Reagent as Chromogen in Highly Sensitive Peroxidase Assays for Enzyme Linked Immunoadsorbent Assay

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Summary: Most of the currently used enzyme immunological assays employ horse radish peroxidase as marker enzyme. A comparison is presented of ABTS (2,2′-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid) and the *Trinder* reagent as chromogens for the detection of small amounts of solid phase peroxidase.

The formation of chromophore using the *Trinder* reagent under the conditions described by *Gallati* (J. Clin. Chem. Clin. Biochem. (1977) *15*, 699–703) reaches a plateau after 3–4 h due to H_2O_2 induced inactivation of the enzyme.

In contrast, with suitable temperature and concentrations of H_2O_2 and ABTS, chromophore production continues in this system for at least 20 h.

In an Enzyme Linked Immunoadsorbent Assay for antibodies to myelin basic protein the use of the ABTS/H_2O_2 substrate system described here gives an assay 14 times more sensitive than the maximum possible with *Trinder* reagent.

Überlegenheit von ABTS über Trinder's Reagenz als Chromogen in hochempfindlichen Peroxidase-Bestimmungen für „Enzyme Linked Immunoadsorbent Assay"

Zusammenfassung: Die meisten der gebräuchlichen enzym-immunologischen Bestimmungen verwenden Meerrettich-Peroxidase als Markerenzym. Hier wird der Einsatz von ABTS (2,2′Azino-di-(3-ethyl benzthiazolin-6-sulfonsäure) als Chromogen zur Darstellung von geringen Mengen gebundener Peroxidase mit dem Einsatz von *Trinder*'s Reagenz verglichen.

Die Bildung von Chromophoren bei der Benutzung von *Trinder*'s Reaganz unter den von *Gallati* (J. Clin. Chem. Clin. Biochem. (1977) *15*, 699–703) beschriebenen Bedingungen kommt nach 3–4 Stunden infolge der durch H_2O_2 induzierten Inaktivierung des Enzyms zum Stillstand. Im Gegensatz dazu führt der Einsatz vor H_2O_2- und ABTS-Konzentrationen und von Temperaturbedingungen, wie sie hier beschrieben werden, im selben System zu einer Chromophorproduktion, die bis zu 20 Stunden andauert.

Der Einsatz von ABTS/H_2O_2 als Substrat in einem ELISA-Test für Antikörper gegen basisches Myelinprotein, wie er hier beschrieben ist, ergibt ein Testsystem, das 14-fach empfindlicher ist als ein vergleichbares, optimal mit *Trinder*'s Reagenz arbeitendes System.

Introduction

Enzyme linked immunoadsorbent assays (ELISA) have found a wide usage (1). Their many advantages over radioimmunoassay include convenience and the longer shelf life of reagents (1). Providing that the reaction conditions are optimised the sensitivity obtained by ELISA may approach that of radioimmunoassay (1). One key factor which determines ELISA sensitivity is the sensitivity of the final enzyme assay stage.

The widespread use of horse radish peroxidase (EC 1.11.1.7) as marker enzyme results from its high turnover number, high stability and ease of coupling to antigens, antibodies and protein A (1, 4). Early chromogens for the detection of peroxidase were insensitive and many were suspected carcinogens (2). ABTS (2,2′-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid), which has no known carcinogenic properties has recently been used successfully in some enzyme immunoassays (1).

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Problems are encountered when attempts are made to extend peroxidase assays for long periods to increase sensitivity (3). Peroxidase is slowly converted by its substrate H_2O_2 into an enzymically inactive form (3). *Gallati* (3) has proposed the use of *Trinder* reagent as a chromogen for the detection of small amounts of peroxidase. He claimed to have defined reaction conditions where inactivation of the enzyme was reduced. However, *Gallati* presented no absolute data on sensitivity (3). No comparison was made with any other chromogen. Furthermore, the *Trinder* assay conditions (3) were not tested on an actual ELISA.

In the present work some of the factors affecting the time course of peroxidase assays were studied. Conditions are described, whereby the peroxidase reaction using ABTS as chromogen, can be made to continue for 20 h and beyond.

The ABTS based assay for peroxidase was then compared with *Trinder* reagent in an enzyme immunoassay for antibodies to myelin basic protein. The use of ABTS, as described, gave an assay at least 14 times more sensitive than the maximum obtainable with *Trinder* reagent.

These observations may be of some interest to those workers who require highly sensitive enzyme immunoassays based on peroxidase.

Materials and Methods

Horse radish peroxidase (Grade 1) and ABTS were obtained from Boehringer, Mannheim, GmbH, West Germany.

Goat antirabbit IgG/peroxidase conjugate was obtained from Miles UK Ltd., Stoke Court, Slough, Berks, U.K.

4-amino-antipyrine was obtained from the Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

Hydrogen peroxide and other laboratory chemicals of Analytical Grade were obtained from BDH Chemicals, Poole, Dorset, U.K.

Peroxidase assays

All peroxidase assays were carried out in 0.1 mol/l phosphate buffer pH 7.0. Reactions were initiated by the addition of 10 µl of an appropriate enzyme dilution to 1 ml of substrate solution. All dilutions of enzyme were made in 0.1 mol/l phosphate buffer pH 7.0 containing 10 g/l bovine serum albumin. ABTS was used at a concentration of 1.8 mol/l throughout.

Trinder reagent was used as described by *Gallati* (3).

ELISA for antibodies to myelin basic protein

Bovine myelin basic protein was used to coat disposable polystyrene tubes. The tubes, containing 1 ml amounts of various dilutions of a rabbit antiserum to myelin basic protein were then incubated for 16 h to allow antibody attachment. Bound rabbit IgG was detected by further incubation in the presence of a goat-antirabbit IgG/peroxidase conjugate, followed by peroxidase assay. 1 ml of the appropriate substrate solution was added to each tube and the tubes were incubated for 20 h at 20 °C in a shaking water bath. The absorbance values at 420 nm were read against an appropriate blank. Full details of this ELISA for antibodies to myelin basic protein will be described in a further communication.

Results

Kinetics of chromophore production using Trinder reagent and ABTS

Figure 1a shows the time course of colour production when a small amount of peroxidase (peroxidase conjugated goat antirabbit IgG) was incubated with H_2O_2/*Trinder* reagent at 37 °C. Conditions were as described by *Gallati* (3). The absorbance at 492 nm reached a plateau after 3–4 h. It then remained constant for long periods. This cessation of enzyme action is due to the formation of an enzymically inactive complex between peroxidase and its substrate H_2O_2 (3).

Figure 1a also shows a time course for the same amount of peroxidase, using the same H_2O_2 concentration (0.8 mmol/l) but replacing the *Trinder* reagent with 1.8 mmol/l ABTS. A similar cessation of enzyme activity takes place as observed with the *Trinder* reagent. In the reaction with ABTS, however, the chromophore is unstable due to a disproportionation reaction (5). Therefore the final absorbance at 420 nm slowly decreases on further incubation.

It was next decided to investigate the parameters affecting the time course of the peroxidase reaction, using ABTS as chromogen. This was to determine whether the reaction could be made to continue over a longer period with a consequent increase in sensitivity.

Effect of temperature on the time course of the peroxidase reaction

Figure 1b shows the time course of the ABTS and *Trinder* assays under similar conditions to figure 1a but carried out at 20 °C instead of 37 °C. Although the initial rates of reaction were reduced by lowering the temperature, enzyme action then continued over a longer period. This is because the temperature reduction has a greater effect in slowing inactivation of the enzyme by H_2O_2 than in slowing the rate of catalysis. The final absorbance at 420 nm at the plateau using ABTS as chromogen was twice the value at 20 °C as at 37 °C.

To determine the optimum temperature at which to carry out at 20 h peroxidase assay several identical ABTS/H_2O_2 substrate mixtures were placed at different temperatures and the reactions initiated by the addition of the same amount of peroxidase to each. The absorbance at 420 nm of each assay was read at various times against a blank which had been incubated at the same temperature (fig. 2).

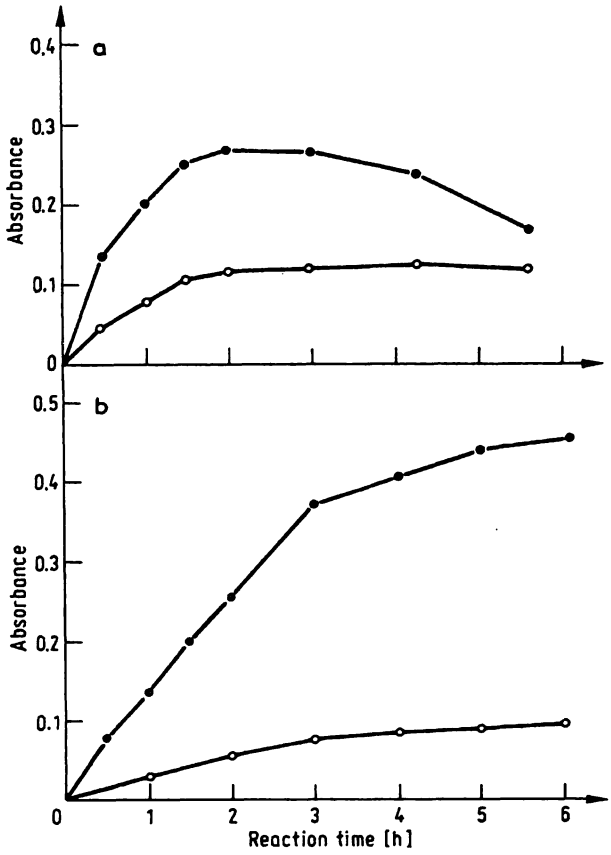

Fig. 1. Time course of peroxidase reaction at different temperatures.
a) at 37 °C
b) at 20 °C
ABTS as chromogen, •——• = absorbance at 420 nm:1.8 mmol/1 ABTS, 0.8 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0.
Trinder reagent as chromogen, o——o = absorbance at 492 nm:2 mmol/l 4-amino-antipyrine, 25 mmol/l phenol, 0.8 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0 (3).
All assays contained the same amount of peroxidase (goat anti rabbit IgG/peroxidase conjugate).

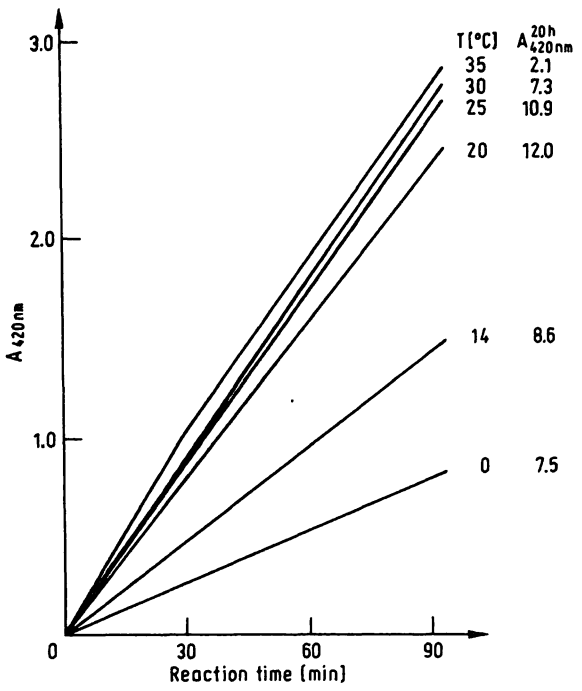

Fig. 2. Time course of peroxidase reaction at various temperatures. The temperatures are given in the first column; the second column indicates absorbance at 420 nm after a 20 h incubation (measured on a 10^{-1} dilution). 1.8 mmol/l ABTS, 0.8 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0. All reactions were initiated with the same amount of peroxidase conjugated antibody.

As expected the initial rate of reaction was reduced as the temperature was lowered. The figures added to figure 2 indicate the final absorbances obtained after a total incubation time of 20 h, and it is apparent that there is an optimum temperature at which to carry out the peroxidase assay for maximum sensitivity. Above 20 °C, H_2O_2 induced inactivation of the enzyme limits the final absorbance. Below 20 °C although inactivation of the enzyme is reduced, this is offset by reduction in the intrinsic rate of catalysis.

Superimposed upon these effects will be any effects of temperature on the stability of the final ABTS chromophore. It is also possible that some loss of enzyme activity is due to simple thermal denaturation and not to reaction with H_2O_2. The present data did not permit a precise kinetic description of these complex events. They did, however, suggest that 20 °C was close to the optimum temperature at which to carry out a 20 h peroxidase assay with ABTS. This temperature was therefore selected for routine use.

Effect of H_2O_2 concentration on the time course of the peroxidase reaction

H_2O_2 concentration was another variable whose reduction it was thought might further offset the inactivation of the enzyme. Figure 3 shows that raising the concentration of H_2O_2 above 0.8 mol/l resulted in accelerated destruction of the enzyme. Reduction of the H_2O_2 concentration to 0.4 mmol/l and 0.2 mmol/l resulted in reactions where colour production continued for 17 h and beyond. At 0.1 mmol/l H_2O_2 the reaction again appeared to plateau prematurely. This was assumed now to be due to depletion of substrate.

This experiment showed clearly that reduction in H_2O_2 concentration prolongs the peroxidase reaction. To determine whether this observation could be put to some practical purpose it was thought necessary to test the ABTS/H_2O_2 assay conditions on an authentic enzyme linked immuno-adsorbent assay.

Effect of H_2O_2 concentration on the sensitivity of an enzyme linked immunoadsorbent assay (ELISA)

The ELISA used was an assay for rabbit antibodies to bovine myelin basic protein. Conditions of the assay are

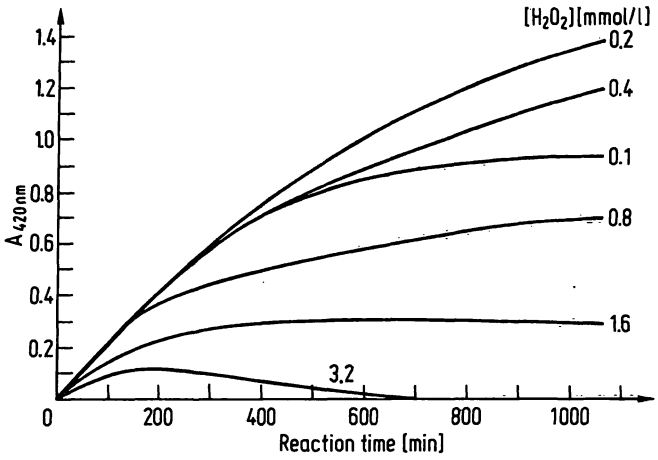

Fig. 3. Time course of peroxidase reaction at various H_2O_2 concentrations.
ABTS concentration 1.8 mmol/l in 0.1 mol/l phosphate buffer pH 7.0.
All reactions were initiated with the same amount of peroxidase conjugated antibody.

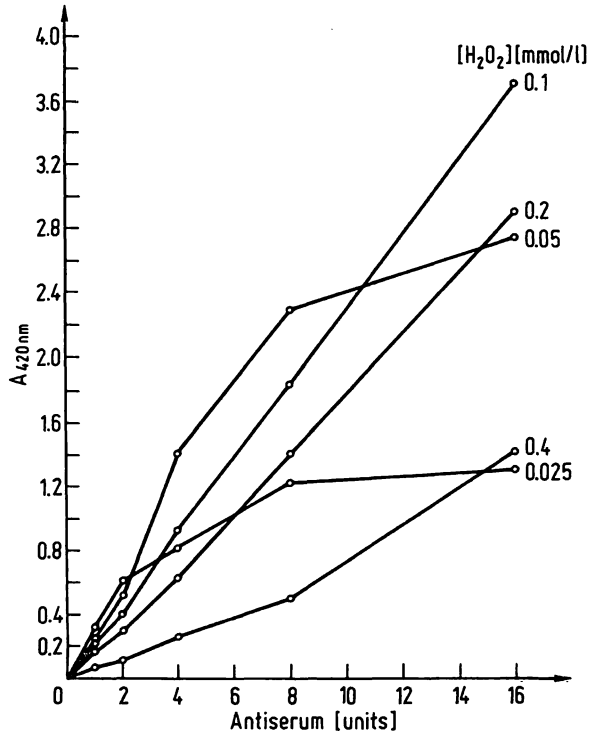

Fig. 4. Effects of H_2O_2 concentration on an ELISA for rabbit antibodies to bovine myelin basic protein. Procedure as described in "Methods". 1 unit of antiserum is defined as 1 ml of a 1 in 256 000 dilution of reference anti MBP serum KK42. The final peroxidase assay is initiated by adding 1 ml of substrate solution to each tube. The tubes are then incubated for 20 h at 20 °C in a shaking water bath before reading the absorbance at 420 nm. The substrate solutions contained 1.8 mmol/l ABTS in 0.1 mol/l phosphate buffer pH 7.0 supplemented with different amounts of H_2O_2.

described briefly in 'Methods'. The experiment (fig. 4) shows that the relationship between absorbance and the amount of antibody is complex and very dependent on H_2O_2 concentration, as expected.

Although the sensitivity at 2 units of antiserum gradually increases as the H_2O_2 concentration is reduced even down to 0.025 mmol/l the range of the assay is then restricted by H_2O_2 depletion at the higher peroxidase activities associated with more antiserum. The H_2O_2 concentration which gave the closest approximate to a linear relationship between absorbance and antiserum amount up to an absorbance at 420 nm = 1.0 was 0.1 mmol/l H_2O_2. Subsequent experiments have shown that the relationship between absorbance and antiserum amount for 0.1 mmol/l H_2O_2 is slightly non linear, with a tendency for the graph to curve towards the absorbance axis.

Comparison of sensitivity obtainable with ABTS and Trinder reagent

Gallati has presented the *Trinder* reagent as a chromogen for the detection of the small amounts of peroxidase as required in ELISA (3). It appeared likely that the ABTS assay devised here was many times more sensitive than possible with the *Trinder* reagent.

Figure 5 shows two identical ELISA titrations where the only difference is the final peroxidase assay. One uses *Trinder* reagent and the conditions described by *Gallati* (3). The other uses 1.8 mmol/l ABTS 0.1 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0 at 20 °C for 20 h. These were the conditions found to be optimal in the present study.

The latter assay was 14 times more sensitive than that published by *Gallati*.

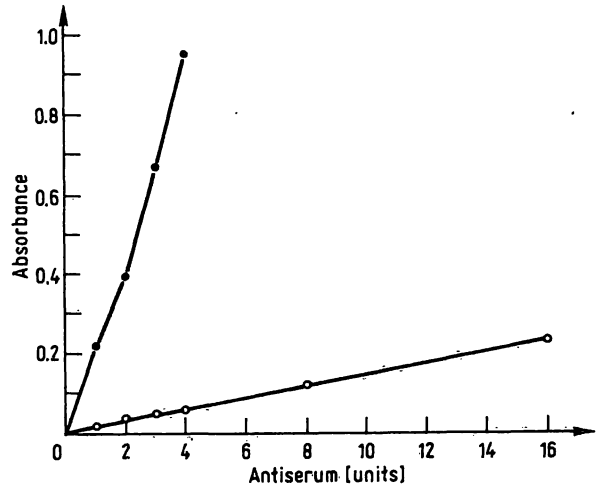

Fig. 5. A comparison of ELISA sensitivity using *Trinder* reagent and ABTS as chromogens. Assay for antibodies to myelin basic protein as in figure 4.
ABTS: 1.8 mmol/l ABTS, 0.1 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0. 20 °C for 20 h, ●——● = absorbance at 420 nm.
Trinder: 2 mmol/l 4-amino-antipyrine, 25 mmol/l phenol, 0.8 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0.
37 °C for 6 h, o——o = absorbance at 492 nm.

Absolute sensitivity of the peroxidase assay

Various known amounts of purified horse radish peroxidase were incubated with ABTS/H_2O_2 as described above. The absorbances at 420 nm after 20 h were read against a suitable blank. Figure 6 shows a concave form similar to that found in the ELISA of figure 4 and figure 5. This suggests that the concave relationship is due to the complexity of the kinetic reaction and is independent of whether the peroxidase is in solution or solid phase. Since an absorbance of 0.02 is still measurable against a low blank this assay should be capable of detecting 0.02 ng of peroxidase.

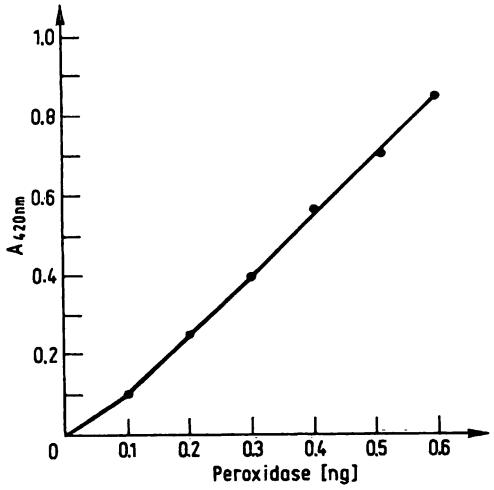

Fig. 6. Relationship between absorbance and amount of peroxidase. Peroxidase (Boehringer Type 1) was added to 1 ml volumes of substrate mixture; 1.8 mmol/l ABTS, 0.1 mmol/l H_2O_2 in 0.1 mol/l phosphate buffer pH 7.0. Tubes were incubated at 20 °C in a shaking water bath for 20 h before reading the absorbances at 420 nm against a blank.

Discussion

These experiments were stimulated by the need to develop a highly sensitive assay for solid phase peroxidase. No references could be found to peroxidase assays where the reaction had been extended beyond one hour. When attempts were made to use ABTS for long term assay the reaction immediately became apparent.

Systematic variation of temperature and H_2O_2 concentration have defined condition where the peroxidase reaction continues for long periods. The conditions described here were tested in an ELISA for antibodies to myelin basic protein and found to give an assay at least 14 times more sensitive than that obtainable with *Trinder* reagent (3).

The enzyme linked immunoadsorbent assay for rabbit antibodies described here uses peroxidase conjugated to antirabbit IgG as the detection reagent. If one makes several assumptions it is possible to calculate the minimum amount of rabbit IgG detectable by such a procedure using the ABTS/H_2O_2 peroxidase assays. These assumptions are:

1. That the first antibody has at least 5 antigenic determinants accessible to second antibody and that these can be occupied simultaneously in the presence of excess second antibody (6).
2. The second antibody is conjugated to peroxidase in a 1:1 molar ratio (7).
3. The conjugated peroxidase retains its full enzyme activity (7).

Making these assumptions it can be calculated that with the present peroxidase assay an absorbance of 0.02 would correspond to 0.015 ng of rabbit IgG.

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